

Mitochondrial functional interactions between frataxin and Isu1p, the iron–sulfur cluster scaffold protein, in *Saccharomyces cerevisiae*

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Abstract Friedreich's ataxia is caused by a deficit in the mitochondrial protein frataxin. The present work demonstrates that in vivo yeast frataxin Yfh1p and Isu1p, the mitochondrial scaffold protein for the Fe–S cluster assembly, have tightly linked biological functions, acting in concert to promote the Fe–S cluster assembly. A synthetic lethal screen on high iron media with the mild G107D *yfh1* mutant has specifically identified Isu1p. Analysis of the cellular phenotypes resulting from pairwise combinations of *yfh1* and *isu1* mutations, and cross-linking experiments in isolated mitochondria provide evidence for a direct interaction between Yfh1p and Isu1p.

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Key words: Fe–S cluster; Frataxin; ISU; Synthetic lethal; Yeast

1. Introduction

Reduced levels of human frataxin cause Friedreich's ataxia (FA), a recessive neurodegenerative disorder often associated with cardiomyopathy [1,2]. An early observation was that frataxin deficiency results in a specific defect of the Fe–S proteins in Friedreich patients [3]. Lack of Fe–S protein activity may be caused by either oxidative damage to labile Fe–S clusters (ISCs) or alteration of the ISC synthesis which has been shown to occur within mitochondria through an ISC machinery strongly conserved during evolution [4]. Although there is good evidence that frataxin plays a role in the antioxidant signalling pathway [5] several recent data argue for a direct role of frataxin in the ISC machinery. First, it has been shown in yeast and transgenic mice that in the absence of mitochondrial iron accumulation the activity of Fe–S proteins is not restored, implying that iron-induced damage is not the primary cause of the ISC defect [6,7]. Second, the synthesis of ISCs is substantially decreased in isolated mitochondria and in mitochondrial extracts of frataxin-deficient or conditionally frataxin-depleted strains [8,9]. Third, recombinant purified frataxins from yeast, bacteria and humans bind iron in vitro [10–12]. Finally, it has recently been reported that in vitro human holo-frataxin binds six to seven iron ions and mediates the transfer of the bound iron to ISU [13], a key component of the ISC machinery. In the presence of iron and sulfur

provided by a cysteine desulfurase [14,15], ISU is used as a scaffold for the transient assembly of a [2Fe–2S] cluster [16,17]. In addition, a very recent report shows that yeast frataxin expressed as a glutathione-S-transferase fusion mitochondrial protein can be purified by affinity chromatography as a complex containing Isu1p and the Nfs1p cysteine desulfurase [18]. These in vitro and biochemical studies require biological in vivo support. Here we report the results of a blind search using a synthetic lethality screen for proteins functionally interacting with yeast frataxin.

2. Materials and methods

2.1. Strains, plasmids and media

Saccharomyces cerevisiae strains are isogenic to W303-1B (*MATα ade2-1 leu2-3,112 his3-11,15 ura3-1 trp1-1*), or are haploid meiotic segregants from crosses between W303-1B and W303-1A (*MATα*) derivatives. The names of the strains are in capital letters (Δ YFH1, G60D ...) and those of the alleles are in italic letters (*yfh1Δ*, *isu1* ...). Mutants A77T, A94P, G107D, P125S, L162F, M21I and E53L were obtained by in vitro hydroxylamine mutagenesis of the centromeric pFL39/YFH1 plasmid [19] followed by transformation of Δ YFH1 strain (*MATα ade2-1 leu2-3,112 his3-11,15 ura3-1 trp1-1 yfh1Δ::Kan^R*) with the pool of mutagenized plasmids and screening for clones unable to grow on 10 mM FeSO₄ at 37°C. All *isu1* alleles, including G60D and M141V, which were obtained by site-directed mutagenesis [20], are borne by the centromeric pRS313 plasmid. All polymerase chain reaction (PCR) products were verified by DNA sequencing.

The Δ ISU1 strain was obtained in strain W303-1B by replacing the coding region and flanking sequences (–61 bp to +289 bp relative to the ATG start codon) by the *URA3* gene. The correct integration of the *URA3* cassette at the *ISU1* locus was confirmed by PCR. Mutants A77T Δ ISU1, A94P Δ ISU1, G107D Δ ISU1, P125S Δ ISU1 and L162F Δ ISU1 were isolated as follows. Firstly, strain Δ YFH1-3C (*MATα leu2-3,112 his3-11,15 ura3-1 trp1-1 yfh1Δ::Kan^R*) was transformed with pFL39 plasmids bearing the *yfh1* mutations, secondly, the transformed strain was crossed with the Δ ISU1 strain, and *yfh1Δ isu1Δ* meiotic segregants harboring a pFL39-borne *yfh1* allele were selected. These mutants have also been transformed with pRS313, pRS313/ISU1, pRS313/M141V, pRS313/G60D-M141V and pRS313/G60D plasmids.

The strains were grown in 2% glucose minimum solid medium, or in 2% raffinose synthetic liquid medium. The media were supplemented with FeSO₄ at the indicated concentration.

2.2. Mitochondria isolation, enzyme activities, Western analysis, ISC incorporation into Yah1p and disuccinimidyl glutarate (DSG) cross-linking

Mitochondria were prepared from cells grown to late exponential phase in synthetic raffinose medium after sphaeroplast lysis [8]. Aconitase and isocitrate dehydrogenase activities were measured by standard procedures. 'Non-heme and non-Fe–S' iron concentration was measured using bathophenanthroline disulfonic acid [21]. Levels of aconitase, Yfh1p, and Isu1p/Isu2p were determined by Western blotting using polyclonal antibodies raised against yeast aconitase and

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Yfh1p, and *Escherichia coli* IscU (a gift from L.E. Vickery, University of California, Irvine, CA, USA). Import of ^{35}S -radiolabeled apo-Yahlp into isolated mitochondria and incorporation of ISC into mature Yahlp were performed exactly as described [8]. Chemical amounts ($\sim 5\ \mu\text{g}$) of purified apo-Yahlp overexpressed in *E. coli* were used in the assay using ^{35}S -cysteine [8]. After mitochondrial lysis, apo- and holo-Yahlp were separated on native acrylamide gels, detected by autoradiography of the dried gels and the signals were quantified.

For cross-linking experiments ^{35}S -Yfh1p and ^{35}S -Isu1p synthesized in vitro (Promega) were imported into mitochondria ($\sim 150\ \mu\text{g}$ protein in 200 μl of import buffer, pH 7.4) [8]. After a 10 min incubation at 23°C, 2 μl of 20 mM DSG solution in dimethylsulfoxide (DMSO) was added to reach a final concentration of 200 μM and 2 μl DMSO was added to mocked samples. After a 30 min incubation at 23°C, cross-linking was quenched by 50 mM Tris-HCl, pH 7.5, and mitochondria were washed twice by centrifugation in 1 ml of a buffer containing 0.6 M sorbitol, 50 mM KCl and 20 mM Tris-HCl, pH 7.4. The mitochondrial suspension was then incubated on ice for 30 min with 200 $\mu\text{g}/\text{ml}$ proteinase K, proteinase K was inactivated with 2 mM phenylmethylsulfonyl fluoride acid and the mitochondria were extensively washed in the same buffer as above.

3. Results

3.1. A synthetic lethal screen identified *ISU1* as a genetic YFH1 partner

Our goal was to identify genes functionally related to *YFH1* by searching extragenic mutations causing lethality on iron media in the presence of a mild *yfh1* mutation. First, we isolated seven *yfh1* mutants with impaired growth on 7 mM FeSO_4 at 37°C (Fig. 1A). M21I and E53L mutations are located in the mitochondrial targeting presequence, and A77T, A94P, G107D, P125S and L162F are scattered in the mature protein. All mutants had mild phenotypes. Their cellular growth at 28°C on 7 mM FeSO_4 was wild-type (or almost wild-type) while that of strain ΔYFH1 was inhibited (Fig. 1A). In A94P, the most affected mutant, the aconitase activity, which reflects ISC machinery activity, was decreased by 50% and mitochondrial iron content was 2.5-fold higher than in wild-type (data not shown). Under these conditions, the mitochondrial iron content of the ΔYFH1 strain was 10 times higher than in wild-type and aconitase activity was less than 15%.

Mutant G107D was chosen to perform the in vivo ethylmethanesulfonate mutagenesis and synthetic lethal screen. This mutant has the mildest phenotype and is modified in a conserved residue whose Gly130 equivalent in humans is re-

placed by a valine in an atypical form of FA [22]. The present work is focused on one of the synthetic lethal mutants obtained on 7 mM FeSO_4 at 28°C. In this mutant the pFL39/G107D plasmid could not be lost, suggesting that it was required for cell survival. The mutated gene causing synthetic lethality was identified by cellular growth restoration on 10 mM iron media at 28°C after transformation with a high copy number genomic library that did not contain the *YFH1* gene. Sequencing of the complementing plasmid inserts revealed the complete sequences of *ISU1* (one insert), and *ISU2* (five independent inserts). Isu1p is the major form, and Isu2p, whose mature form is quite similar to Isu1p (85% amino acid sequence identity), is not detectable in cells grown to stationary phase [23]. Although neither *ISU1* nor *ISU2* are essential genes, the double *isu1* Δ *isu2* Δ deletion is lethal [23–24]. The *isu1* and *isu2* genes from the synthetic lethal mutant were cloned by gap-repair and sequenced. No mutation was present in *ISU2*, which behaves as a multicopy suppressor, while two amino acid modifications G60D and M141V were identified in *isu1*.

To determine the contribution of each *isu1* mutation to the increase in iron sensitivity, *ISU1*, G60D, M141V and G60D-M141V alleles were cloned in the pRS313 centromeric plasmid in a wild-type *YFH1* context, and compared with an *isu1* deletion. The M141V mutation did not confer any phenotype, while the G60D mutation was sufficient for slower cellular growth on 4 mM FeSO_4 (Fig. 1B). Curiously, the G60D-M141V mutant was sicker than the ΔISU1 mutant, indicating that the G60D-M141V allele is not silent.

3.2. G107D *yfh1* and *isu1* mutations have synergetic deleterious effects on ISC assembly

Although mutants carrying either *yfh1* Δ or *isu1* mutation alone (*isu1* Δ , G60D or G60D-M141V) were viable, tetrad analysis showed that the combination of both *yfh1* Δ and *isu1* mutations in the same strain was lethal (~ 120 tetrads). In contrast, spores containing *yfh1* Δ and *isu2* Δ alleles were readily obtained. This synthetically lethal trait underlines the tight functional link between Yfh1p and Isu1p, and indicates that Isu2p cannot substitute for Isu1p in a strain that has no frataxin.

However, spores containing *yfh1* G107D and *isu1* Δ mutations together were viable. Nevertheless, although mutants harboring the *isu1* deletion or G107D *yfh1* mutation alone

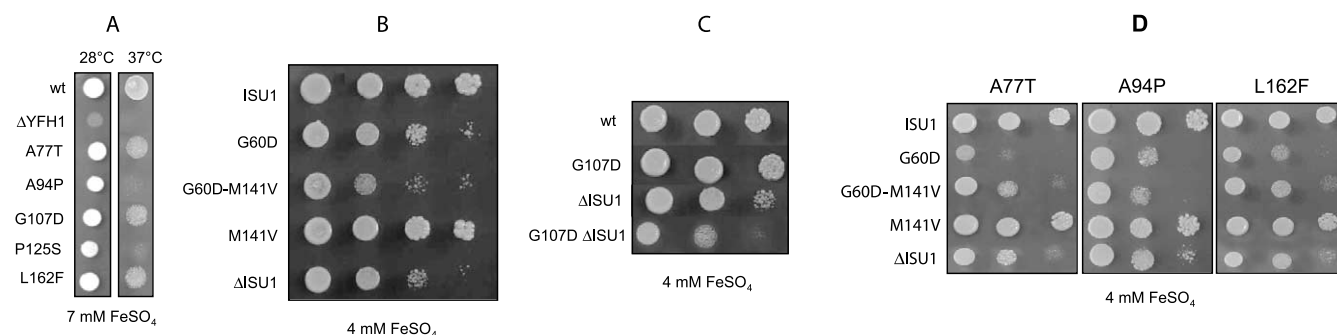


Fig. 1. Cellular growth phenotypes on iron-containing glucose minimum media. A: *yfh1* mutants were incubated on 7 mM FeSO_4 for 3 days at 28°C or 37°C. B: Twenty-fold serial dilutions of *isu1* Δ cells harboring the pRS313 plasmid-borne *ISU1*, G60D, M141V, G60D-M141V alleles or empty pRS313 plasmid (ΔISU1). C: High iron sensitivity of G107D ΔISU1 mutant compared to G107D or ΔISU1 mutants (serial dilutions). D: Synergetic effects in mutants combining *yfh1* and *isu1* mutations (serial dilutions). The *yfh1* Δ *isu1* Δ strains have been transformed with both pFL39 plasmids harboring the different *yfh1* mutations and pRS313 plasmids, either empty (ΔISU1), or harboring *ISU1*, G60D, G60D-M141V, or M141V mutations. On top of each panel the *yfh1* mutation is indicated and the combination with *isu1* mutations is shown on the left.

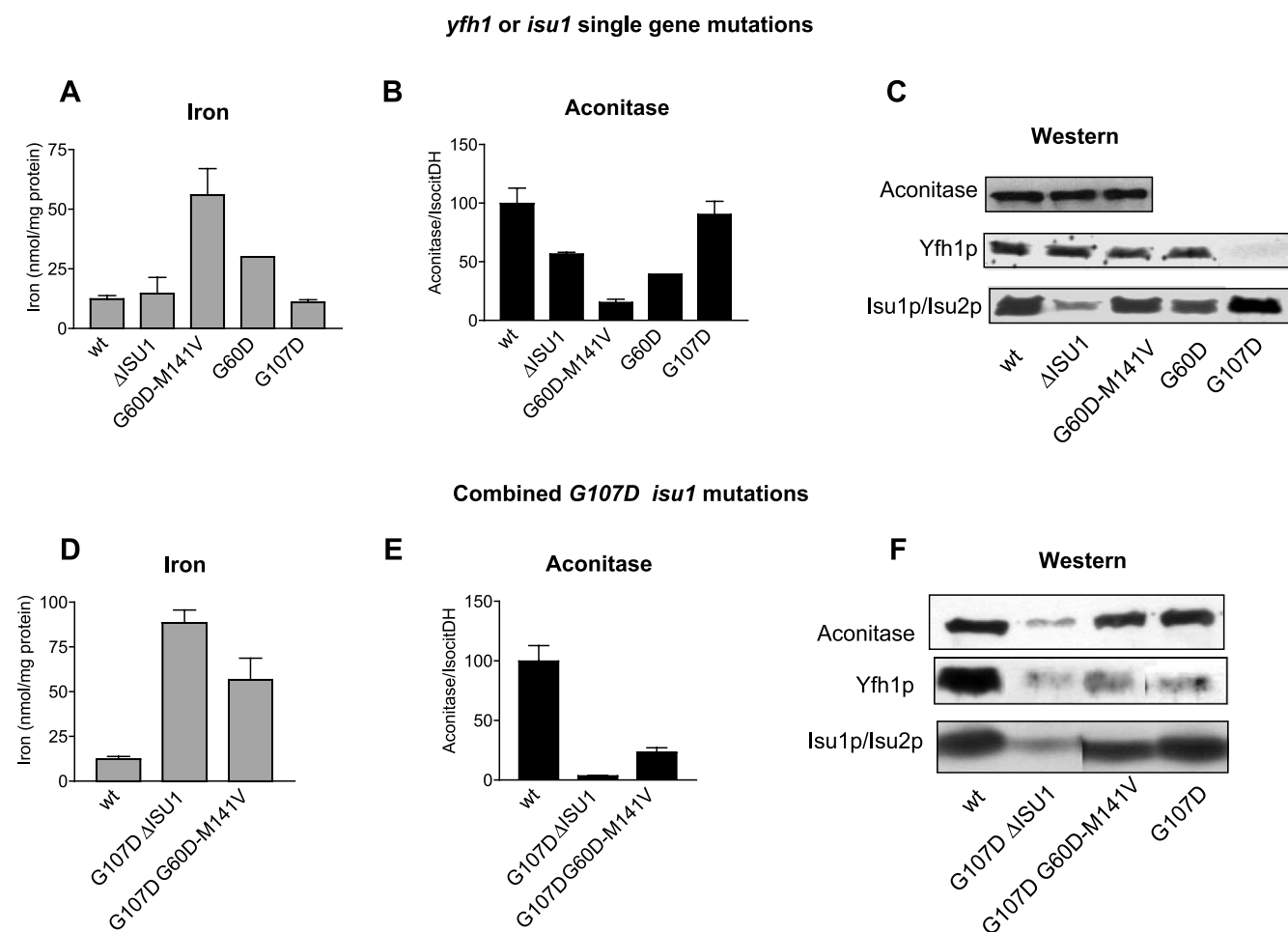


Fig. 2. Synergetic deleterious effects on mitochondrial iron content and aconitase activity in the doubly mutated strain G107D Δ ISU1 compared to the single gene mutation strains G107D and Δ ISU1. Mitochondria were isolated from cells grown in raffinose medium in the presence of 5 μ M FeSO₄. Panels A–C illustrate single gene mutation strains, and panels D–F illustrate strains mutated in both *yfh1* and *isu1* genes. The wild-type strain is W303-1B. The values represent the average of six measurements from three independent cultures and can be compared directly in all mutants. The average mitochondrial iron content of wild-type is 12 nmol/mg protein.

were not severely affected, the combination of both mutations in the same strain led to severe phenotypes in the presence of 4 mM FeSO₄ (Fig. 1C). To better understand the functional link between Isu1p and Yfh1p we compared the biochemical properties of mutants carrying either one or two mutated genes. The mitochondrial iron content and aconitase activity of the G107D *yfh1* mutant in a wild-type *ISU1* context were quite similar to that of the parental strain (Fig. 2A,B). We also measured the incorporation rate of ISCs into the mitochondrial Yfh1p ferredoxin imported into isolated mitochondria. The apo- and holo-forms of Yfh1p can be distinguished by their mobility in native gel electrophoresis. Fig. 3 shows that the conversion rate of the apo- to holo-form was not affected in the G107D mutant, compared to the strong decrease observed in the Δ YFH1 mutant.

Surprisingly, G107D Yfh1p levels estimated by Western blot analysis were very low, though detectable (Fig. 2C). All mutants except P125S (data not shown) had low Yfh1p levels, suggesting a reduction in frataxin stability. This is in agreement with the thermosensitive phenotype of the mutants and the prediction that the mutated residues play a role in the structure of the protein. It has been shown that human G130V frataxin is also unstable [25–26]. Moreover, purified

wild-type Yfh1p compared to *E. coli* and human frataxins has an unusually low melting temperature [11] so that any mutation in the protein could increase its instability. Our data led to the conclusion that minute amounts of frataxin are sufficient to support ISC assembly in mitochondria.

The Δ ISU1 mutant had slightly increased mitochondrial iron content and its aconitase activity was decreased by ~50% (Fig. 2A,B). Therefore, in the Δ ISU1 mutant the low amounts of Isu2p (20% of Isu1p) support ISC synthesis at a rate which is not optimal but still substantial.

However, when *isu1* Δ and G107D *yfh1* mutations were put together the mitochondrial iron concentration was considerably increased and aconitase activity was extremely low (Fig. 2D,E). Aconitase protein levels were strongly reduced (Fig. 2F). Low levels of aconitase were also observed in our Δ YFH1 strain grown in glucose medium (data not shown). They correlate with severe in vivo phenotypes and are not the result of decreased *ACO1* transcript levels. A likely explanation is that under extremely low ISC assembly conditions aconitase is mainly under its apo-form, a protein state recognized and degraded by the proteases in charge of the mitochondrial protein quality control [27].

Altogether, these data show *yfh1* and *isu1* mutations act

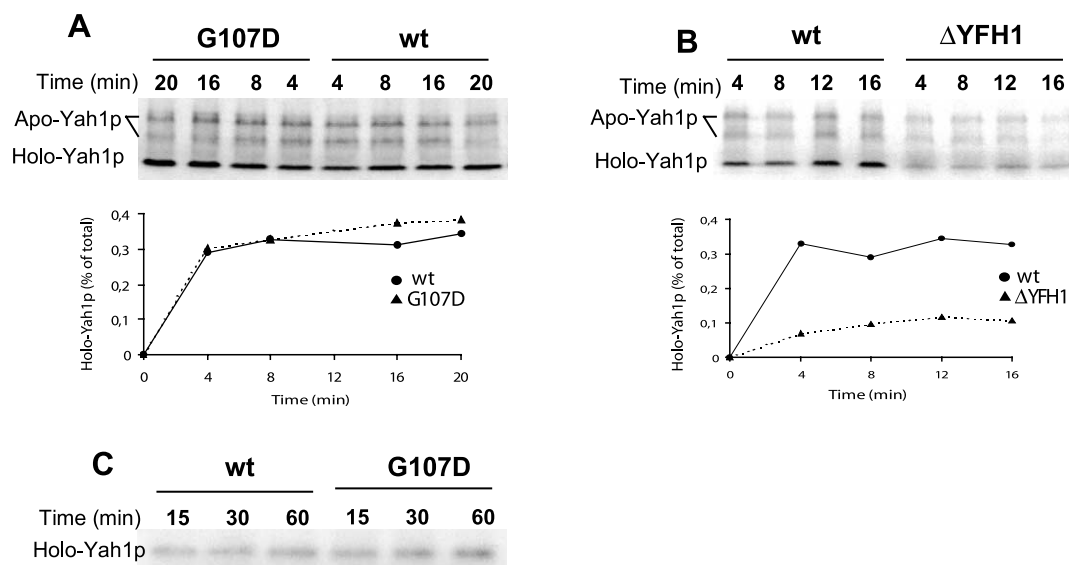


Fig. 3. Incorporation of ISC into mature Yah1p ferredoxin imported into energized isolated mitochondria of wild-type, G107D and $\Delta YFH1$ strains grown in raffinose medium. Import of apo-Yah1p into mitochondria determined by SDS-PAGE was identical in all strains (not shown). A: Incorporation of ISC into mature ^{35}S -labeled Yah1p. The amount of holo-Yah1p was expressed as the ratio of holo-Yah1p to apo- plus holo-Yah1p. B: Incorporation of $\text{Fe-}^{35}\text{S}$ cluster into mature Yah1p present in chemical amounts in the assay. The ISC was radiolabeled in mitochondria as reported [8] with ^{35}S -cysteine added to the assay.

synergically. Low levels of frataxin have no significant consequence on ISC synthesis in a wild-type *ISU1* context. Low levels of Isup have only a moderate effect in a wild-type *YFH1* context. However, the combination of low levels of both proteins has dramatic effects. This indicates that frataxin becomes essential when Isup levels are limiting, and acts in concert with Isup to promote ISC assembly.

The situation was more complex with the G60D-M141V allele. In a wild-type *YFH1* context this double mutation had more dramatic effects than the *isu1* Δ allele (Fig. 2A,B). This severe phenotype is most likely the result of the wild-type expression level of the non- (or poorly) functional G60D-M141V Isup (Fig. 2C) competing in a deleterious manner with the functional Isu2p. Curiously, ISC metabolism was less affected in G107D G60D-M141V than in G107D $\Delta ISU1$ strain (Fig. 2D,E). We have currently no explanation for this observation, which, however, suggests that when G60D-M141V Isup and G107D Yfh1p are together in the mitochondria, they influence the activity of each other.

3.3. Synthetic lethality of *isu1-1* and *yfh1* mutations on iron-containing media is not correlated with *yfh1* mutant phenotype severity

To determine whether the severe phenotype of the G107D $\Delta ISU1$ mutant was allele specific, we generated a set of *yfh1* *isu1* mutants containing pairwise combinations of G60D, G60D-M141V *isu1* or *isu1* Δ mutations and A77T, A94P, P125S or L162F *yfh1* mutations, respectively. In all pairwise combinations, cellular growth sensitivity to iron was substantially increased compared to single gene mutations. However, there was no direct relationship in the severity of the phenotypes obtained with one or two mutated genes (Fig. 1D). A specifically severe phenotype was observed when the G60D *isu1* mutation was combined with the mild A77T and L162F *yfh1* mutations. Cellular sensitivity to iron was even more pronounced than for G60D *isu1* A94P (or P125S) *yfh1* combination, although alone these *yfh1* mutations were much

more severe. Moreover, A77T G60D and L162F G60D mutants were sicker than A77T G60D-M141V and L162F G60D-M141V mutants, respectively, although when alone, G60D-M141V *isu1* was more severe than G60D *isu1*. Therefore, although the mutant phenotypes can roughly be explained by low levels of Yfh1p, the more subtle differences observed between different combinations of *yfh1* and *isu1* mutations suggest that the function of Yfh1p is influenced by the mutation(s) in Isup, and vice versa, as a result of a physical interaction between the two proteins.

3.4. A cross-linking product of same mobility whose formation depends on functional ISC machinery is shared by Yfh1p and Isup imported into isolated mitochondria

^{35}S -radiolabelled Yfh1p and Isup were imported into isolated mitochondria, and mitochondria were treated with DSG, a non-cleavable cross-linking agent penetrating the mitochondrial membranes. A ~ 43 kDa cross-linking product was shared by imported ^{35}S -Yfh1p and ^{35}S -Isup in wild-type mitochondria (Fig. 4A). It was absent in $\Delta YFH1$ and G107D mitochondria containing imported ^{35}S -Isup (Fig. 4A,B), or in $\Delta ISU1$ mitochondria containing ^{35}S -Yfh1p (Fig. 4C). These data suggest that the 43 kDa product contains cross-linked Yfh1p and Isup, an interpretation supported by the observation that cross-linking did not occur when endogenous frataxin or Isup were either absent ($\Delta YFH1$), or in too low amounts (G107D, $\Delta ISU1$) to have some chance of interacting with the ^{35}S -radiolabelled imported protein. Curiously, the apparent mobility of the cross-linking product (43 kDa) was substantially slower than that of each of the two species (apparent mass of 20 kDa for Yfh1p and 15 kDa for Isup). This discrepancy might result from abnormal mobility caused by cross-linking. Another hypothesis would be that the ~ 43 kDa product results from cross-linking between a Yfh1p monomer and an Isup dimer. In several organisms, holo-Isup/Isu are homodimers [28–29]. Surprisingly, the cross-linking product was not detectable in

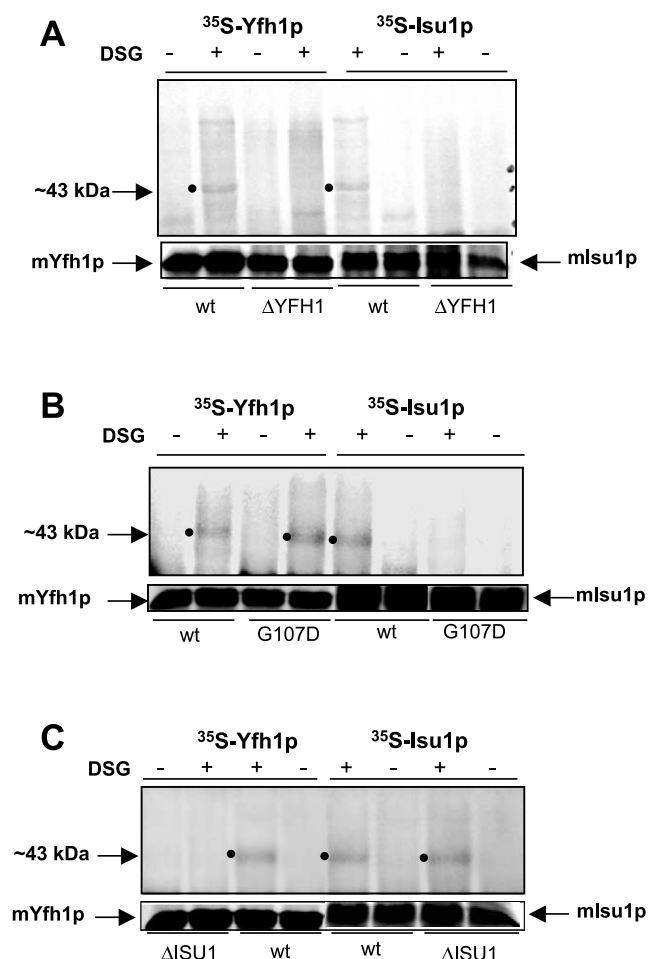


Fig. 4. DSG cross-linking of imported ³⁵S-Yfh1p and ³⁵S-Isu1p in isolated mitochondria. DSG cross-linking in intact mitochondria was performed as described in Section 2. Panels A–C illustrate cross-linking in ΔYFH1, G107D and ΔISU1 mitochondria, respectively, compared to wild-type. The signs – and + refer to mocked and DSG-treated samples, respectively. The picture shows mature ³⁵S-Yfh1p (mYfh1p) and ³⁵S-Isu1p (mIsu1p) in a 13% acrylamide gel, and cross-linking products (upper part) in 10% (A) and 12% (B,C) acrylamide gels. Cross-linking products are indicated by closed circles. Autoradiography exposure length was 3 days for the mature product and 15 days for the cross-linking product. The molecular weights have been estimated using standard molecular weight markers.

ΔYFH1 mitochondria containing ³⁵S-Yfh1p. We believe that the absence of the 43 kDa product in ΔYFH1 mitochondria reflects impaired ISC assembly. This interpretation is supported by the observation that in G107D mitochondria that have a functional ISC machinery, though small amounts of endogenous Yfh1p, the cross-linking product is made in the mitochondria containing imported ³⁵S-Yfh1p (Fig. 4B).

4. Discussion

Our blind search for Yfh1p partners has identified Isu1p and no other member of the ISC machinery. Therefore, we can conclude that Isu1p is most likely the major partner of frataxin in mitochondria.

Recent in vitro studies have shown that an iron-bound form of human frataxin forms a high affinity complex with human ISU1 and is able to mediate the transfer of iron to ISU [13].

Moreover, very recently it has been shown that Yfh1p expressed in mitochondria as a glutathione-S-transferase fusion protein can be purified by affinity chromatography as a complex composed of Isu1p and Nfs1p [18]. These in vitro and biochemical data were based on the a priori assumption that frataxin and ISU interact. Our present work gives the biological support that was missing, and also provides further insight into the relationship between Yfh1p and Isu1p.

A cell that has no frataxin and low levels of Isu2p is not viable, and a cell that has low levels of frataxin and Isu2p is very sick. However, in a wild-type *ISU1* context very low levels of frataxin are sufficient to support efficient ISC assembly, and cells without frataxin are still able to synthesize ISCs though much less efficiently [8]. Thus, in yeast frataxin does not play an essential role as long as Isu1p is not in limiting amounts. This suggests that frataxin assists Isu1p for an optimal function. Here, it must be stressed that the yeast model cannot be applied directly to humans or mice, for which frataxin is essential [2,7]. The interallelic interactions observed in pairwise combinations of *yfh1* and *isu1* mutations are strongly suggestive of physical interactions between the two proteins, and conclusions of this in vivo approach are still strengthened by *in organello* experiments strongly suggesting cross-linking between Yfh1p and Isu1p in intact mitochondria treated with DSG. These data support the in vitro finding that frataxin can mediate the transfer of iron to ISU [13]. However, since total loss of frataxin in glycerol-grown cells only partially affects ISC synthesis [8,30], this implies that another mitochondrial source of iron is available, which is still to be discovered.

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